

Toxicity of FireMaster FF-1 and 2,2',4,4',5,5'-Hexabromobiphenyl in Cultures of C3H/10T 1/2 Mammalian Fibroblasts

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A procedure for purifying 2,2',4,4',5,5'-hexabromobiphenyl (HBB) from FireMaster FF-1 in gram amounts by crystallization is presented. Following purification, the structural assignment of HBB was made by using proton and carbon-13 nuclear magnetic resonance (NMR) spectroscopy, elemental analysis, and mass spectroscopy (MS). The growth of C3H/10T 1/2 cells treated with 5, 37, 75, and 150 μg of HBB and FF-1 per milliliter of medium was measured at 4, 8, and 13 days following treatment. FF-1 was more toxic at 37 and 75 $\mu\text{g}/\text{ml}$ at both 4 and 8 days, but the same at 13 days. At 150 $\mu\text{g}/\text{ml}$ cell growth was completely inhibited by both compounds. Growth of cells was stimulated at 5 $\mu\text{g}/\text{ml}$, by HBB at 4 and 8 days, and FF-1 at 8 and 12 days. HCB was compared with HBB and FF-1 for cell growth toxicity at 37 and 75 $\mu\text{g}/\text{ml}$. At 75 $\mu\text{g}/\text{ml}$, HCB was more toxic than HBB and FF-1 during the entire time period. At 37 $\mu\text{g}/\text{ml}$, HCB was more toxic than HBB and FF-1 at 4 and 8 days. Cells seeded at high densities and treated with HBB for three days lost the high degree of postconfluence inhibition of cell division observed in control cultures. Cells treated with FF-1 for three days did not adhere well to the plastic growth surface. Ultrastructural features of the HBB- and FF-1-treated cells included decreased surface villi and increased lysosomes relative to the control cells.

Introduction

Interest in the toxicity of polybrominated biphenyls has arisen as an aftermath of the widespread contamination of food destined for human consumption (1). HBB has been demonstrated by Norström et al. (2) and Sundström et al. (3) to be the major component of the FireMaster products. The use of cultured cells provides a sensitive assay to detect relative chemical toxicities, particularly at low and chronic levels (4), without the variables of absorption, excretion, and transport. Cultured cells have been used to study the toxicity of a variety of compounds, including the food additive butylated hydroxytoluene (4), and polychlorinated biphenyls (PCB) (5). For this study, we used C3H/10T 1/2

mouse embryo fibroblasts which demonstrate a strict growth pattern, remarkable postconfluence inhibition of cell division and have been well characterized (6, 7).

Materials and Methods

Chemicals and Spectral Analysis

A 100-g portion of FF-1 (Michigan Chemical Corporation) was placed in a 3-liter Erlenmeyer flask in a well-vented hood. Reagent grade acetone (200 ml) was added. The mixture was stirred magnetically at room temperature for 2 hr. The undissolved solid material was collected on a Büchner funnel and air-dried; 60 g of the solid material was recovered and transferred back to the flask. A minimum amount of hot acetone was used to dissolve all of the solid. The resultant acetone solution was heated on a steam bath until precipitates appeared. The cooled solution was filtered, and the crystals

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washed with ice-cold acetone, dropwise. The crystalline materials were redissolved in hot acetone and decolorized with activated charcoal, followed by filtration through Celite. The clear filtrate was concentrated and left at room temperature overnight. Needlelike crystals were collected, washed with 20 ml of ice-cold acetone, and dried under high vacuum for 6 hr. HBB (25.4 g) with 98% purity was obtained in this manner.

Proton NMR spectra were determined using CDCl_3 with TMS as an internal standard on a Varian EM-390 NMA NMR spectrometer. The carbon-13 NMR spectrum was obtained with a Bruker HX-

90E MHz Fourier Transform NMR spectrometer. Elemental analysis was performed by Galbraith Lab. (Knoxville, Tenn.) The mass spectrum was obtained with a Hewlett-Packard 5982 A gas chromatograph-mass spectrometer via direct insertion probe with an ionization voltage of 70 eV.

HCB was synthesized by the method of Hutzinger and Safe (8).

Cell Cultures

Cells were maintained according to the protocol outlined by Reznikoff et al. (6). Eagle's basal medium (Grand Island Biological Co.; GIBCO) supplemented with 10% heat-inactivated fetal calf serum (GIBCO) without antibiotics was utilized. Cells were passaged and treated in a verticle laminar flow hood (Germfree Lab., Miami, Fla.) and incubated at 37°C in a humidified incubator (Lab-line Inst., Melrose Park, Ill.) containing 5% CO_2 in air. Tests were performed and found negative for PPLO organisms.

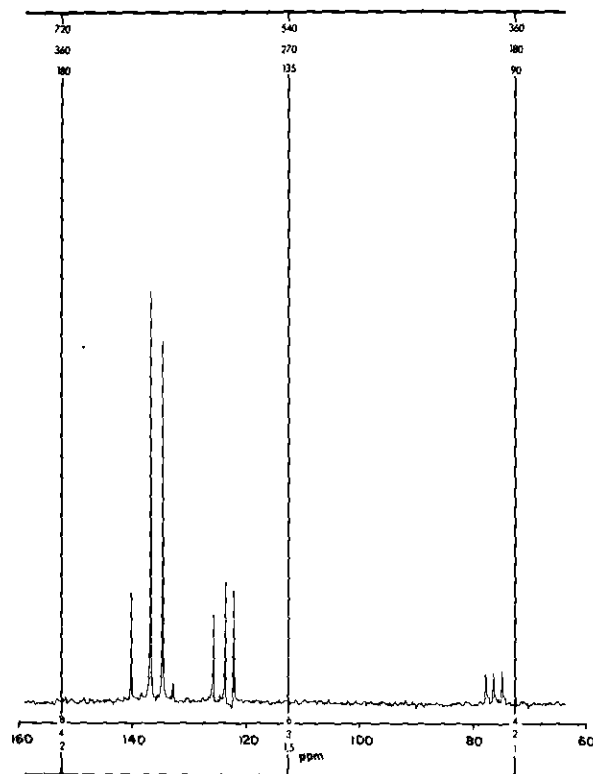


FIGURE 1. ^{13}C NMR spectrum of HBB in CDCl_3 (TMS as internal standard).

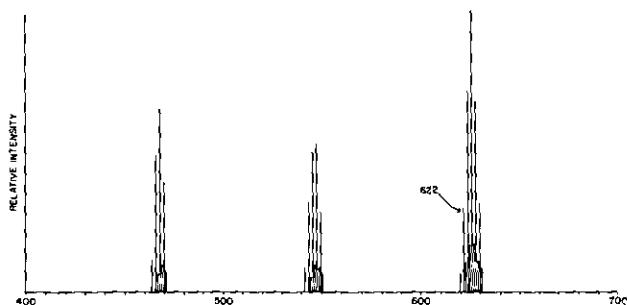


FIGURE 2. Mass spectrum of HBB (m/e of molecular ion identified).

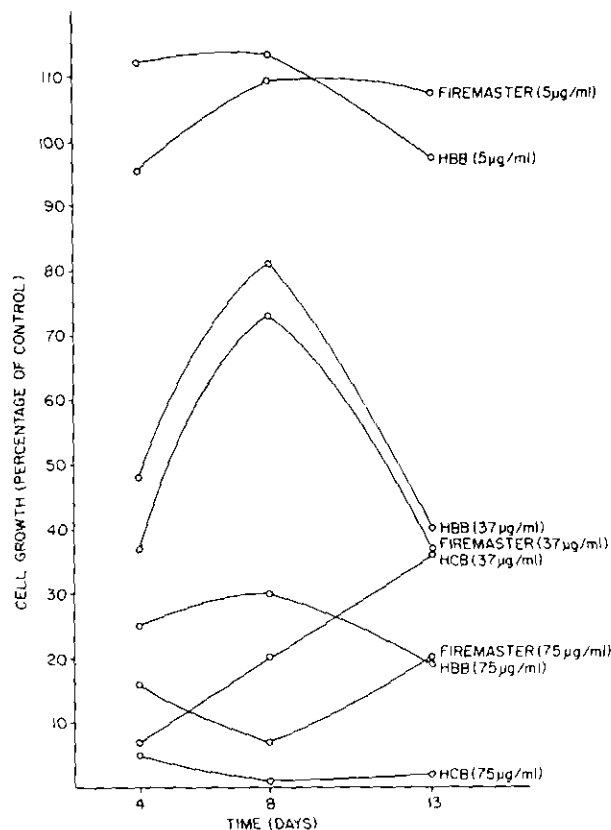


FIGURE 3. Growth of C3H/10T 1/2 cells following treatment. Cells were plated in Falcon culture trays and treated with various concentrations of HBB, FF-1, or HCB as depicted. Replicate cultures were counted at 4, 8, or 13 days. Each point represents an average of two determinations.

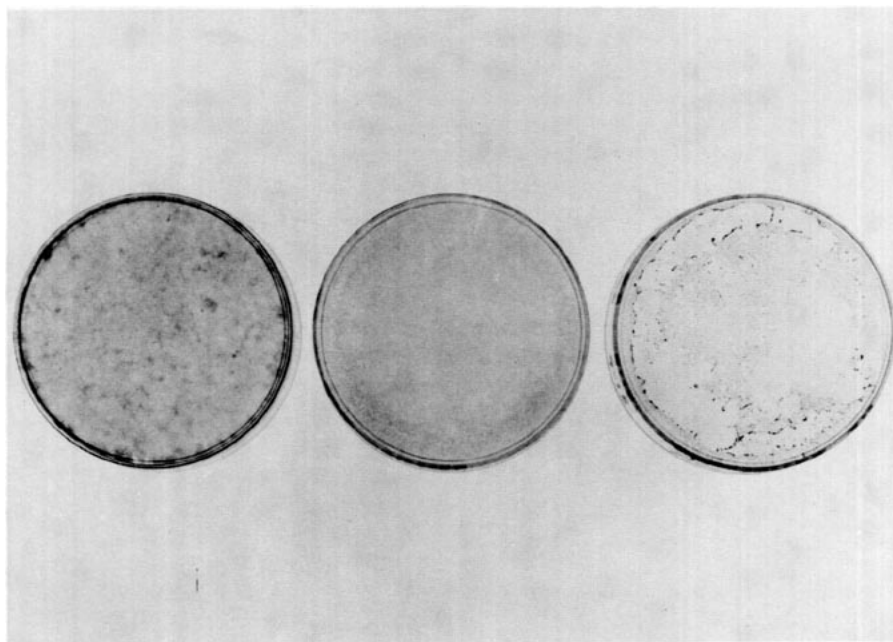


FIGURE 4. C3H/10T 1/2 Cells Treated with (left) 50 µg HBB/ml medium, (center) control medium, or (right) 50 µg FF-1/ml medium. Cells were incubated for 3 days in 60-mm Falcon culture dishes, at which time the medium was replaced with fresh medium containing no experimental compounds. After an additional 4 days, the cells were fixed and stained with Giemsa. Note that the cells form an even layer in the control culture. In the HBB treated culture, cells form areas of increased cell density. In the FF-1 treatment, note the areas where cells are absent.

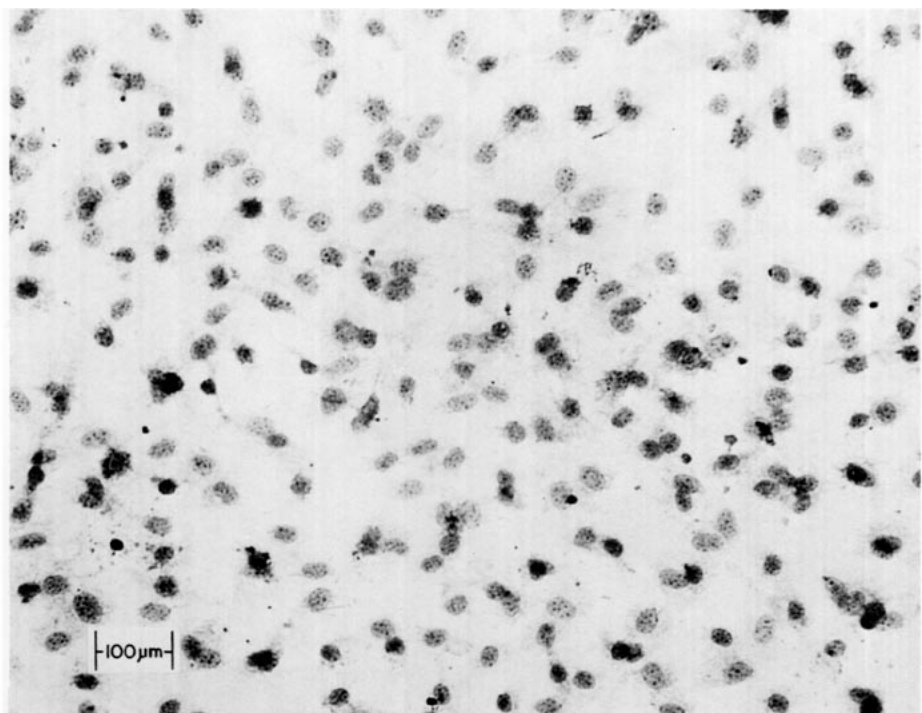


FIGURE 5. Light micrograph of HBB-treated cells pictured in Figure 4. Focal areas of increased cell density, frequently with overlapping nuclei, are observed. Cellular debris is present.

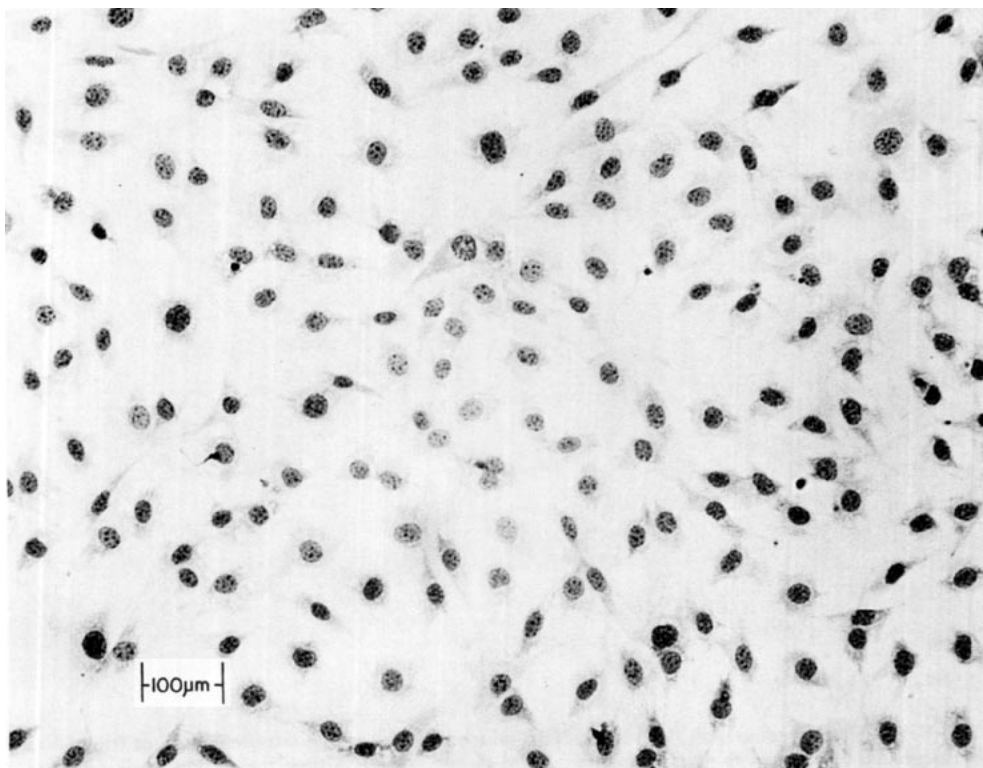


FIGURE 6. Light micrograph of control cells pictured in Figure 4. Note high degree of postconfluence inhibition of cell division with marked separation of cell nuclei.

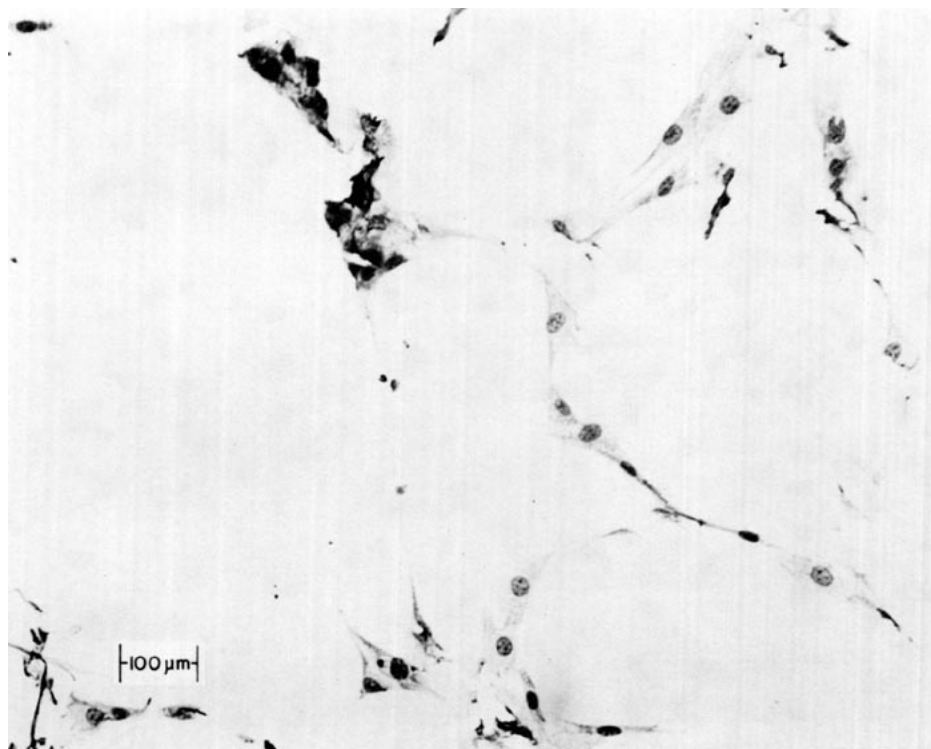


FIGURE 7. Light micrograph of FF-1-treated cells pictured in Figure 4. Cell attachment to the plastic is impaired.

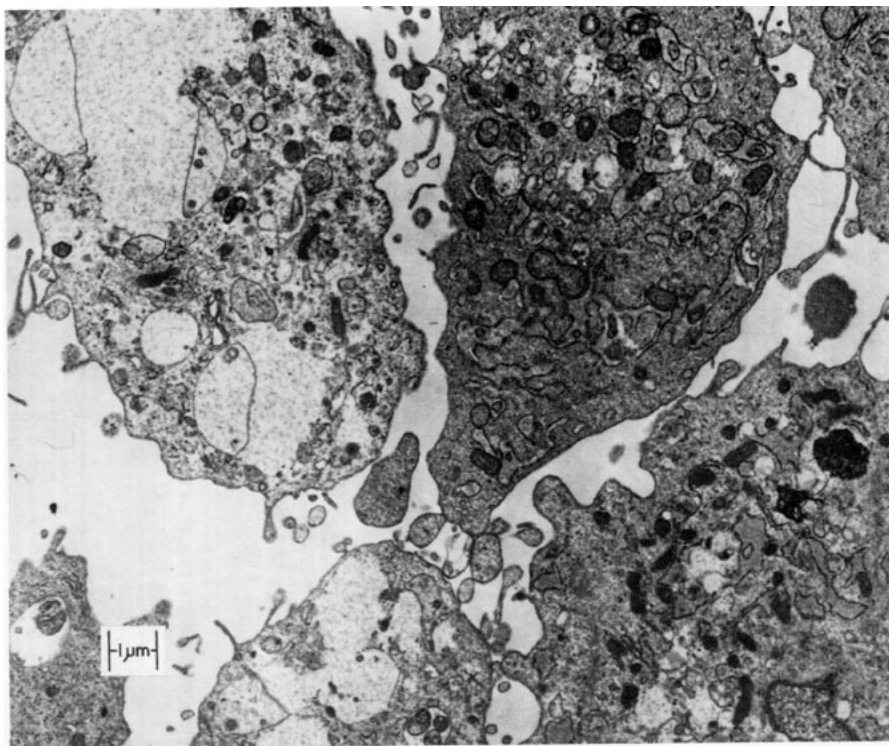


FIGURE 8. Electron micrograph of control cells (treatment B used, see Materials and Methods). Note the markedly irregular and villous cell surfaces, abundant free ribosomes, and sparsity of endoplasmic reticulum. Uranyl acetate stain.

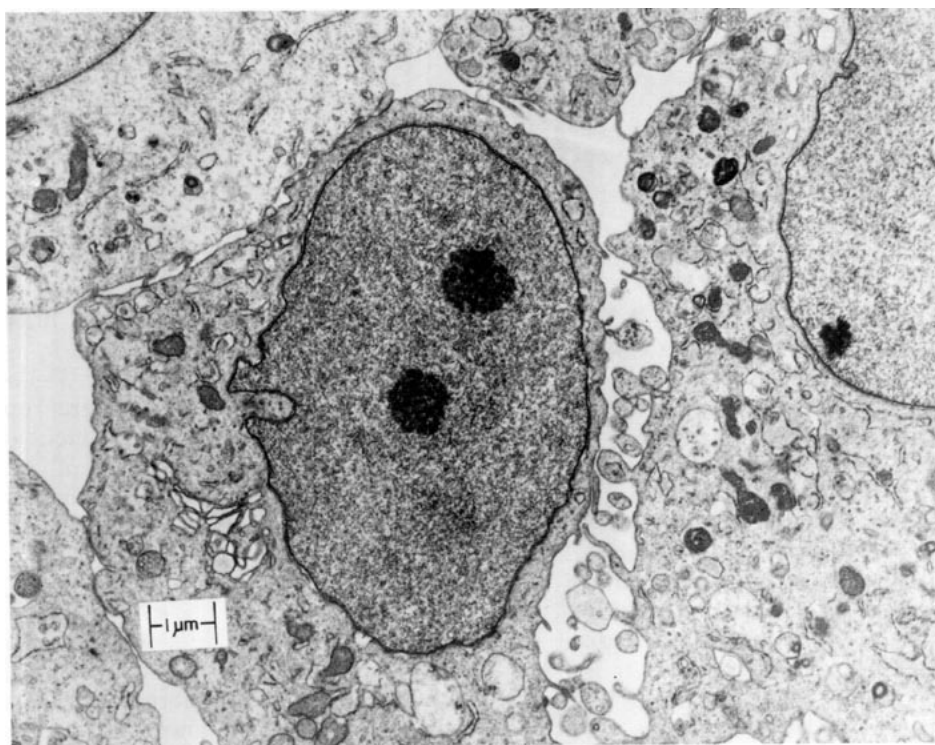


FIGURE 9. Electron micrograph of cells treated with HBB (treatment B, see Materials and Methods). Note the abundant lysosomes and dilated endoplasmic reticulum. The surface of these cells contain fewer villous projections compared to control cells. Uranyl acetate stain.

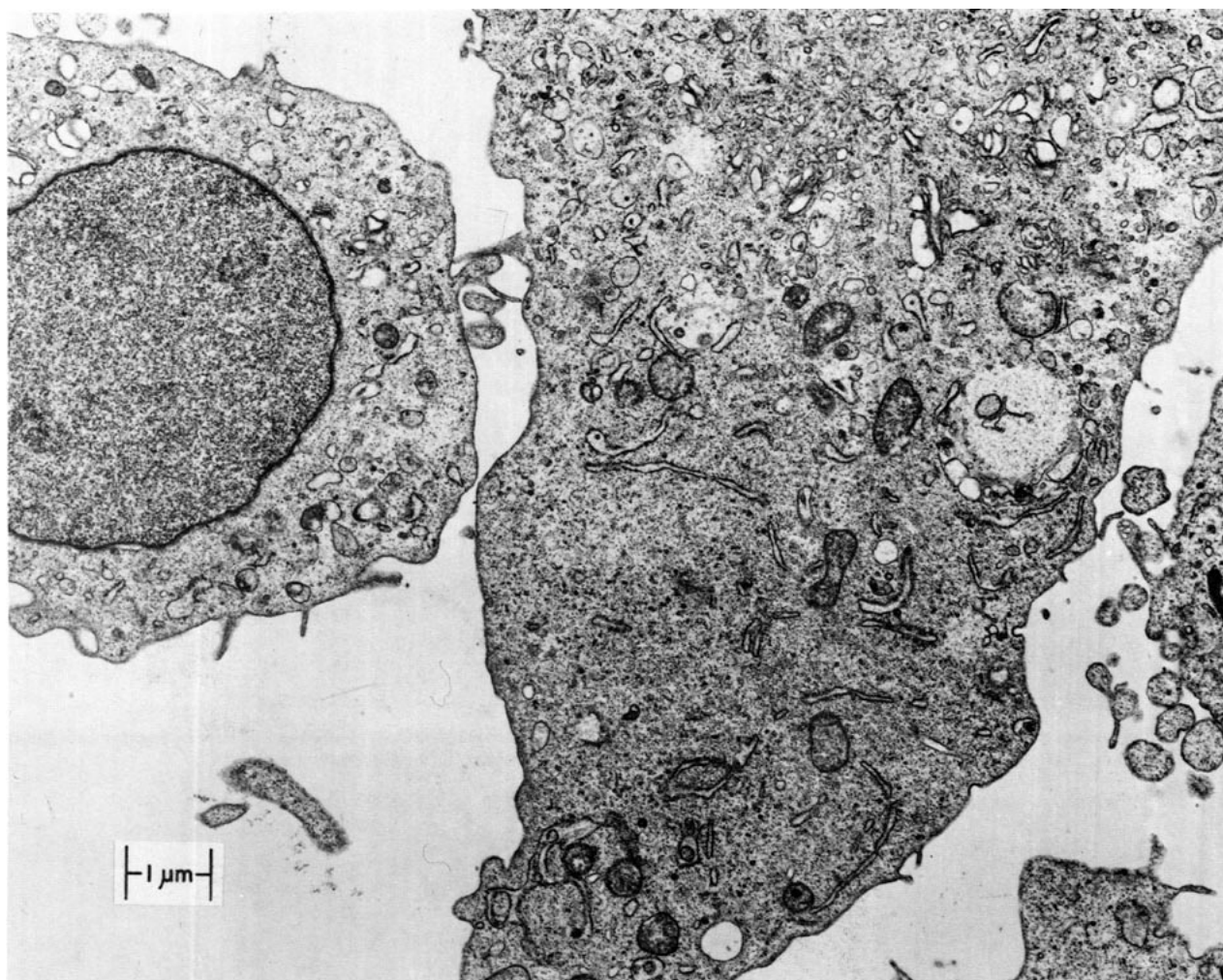


FIGURE 10. Electron micrograph of cells treated with FF-1 (treatment B, see Materials and Methods). Note the abundant cytoplasm with Golgi structures and increased endoplasmic reticulum with closely associated free ribosomes. The smooth cellular surfaces contain few villous projections. Uranyl acetate stain.

Treatment A. The growth of C3H/10T 1/2 cells was tested in the presence of FF-1, HBB, and HCB. Cells were plated in 24-well Dispo-trays (Falcon Plastics) at a density of 3000/cm² growing area. On the following day, the medium was exchanged for medium containing the experimental compounds. The chemicals were diluted with acetone to give concentrations of 15 mg/ml, which in turn was diluted in the medium to give 5, 37, 75, and 150 μg/ml. Control cultures were treated in a similar manner with equivalent amounts of acetone. Following treatments culture dishes were incubated undisturbed and counted at 4, 8, or 13 days. At those times the cells were washed with citrate buffer and treated with 0.25% trypsin (GIBCO) for 5 min. The dislodged cells were counted using a Model B Coulter Counter (Coulter Electronics, Hialeah, Fla.)

Treatment B. Cells were plated at 10,000/cm², and 50 μg/ml FF-1 or HBB was added in a similar manner as described for treatment A. Following a 3-day incubation, the chemicals were removed, fresh medium added, and the cells incubated for another 4 days. At this time samples of cells were prepared for electron microscopic analysis and others for light microscopy.

Microscopy

Examination of living cells was performed by using a Wilt phase contrast microscope (Daigger Co., Chicago). Light photomicrographs were made with Kodak Panatomic-X film with a Zeiss photomicroscope. Cells examined by photomicrography were first fixed in absolute methanol for 15 min, then stained for 1 hr with a 5% solution of Giemsa.

Cells examined using electron microscopy (EM) were first treated with 0.25% trypsin, washed with phosphate buffer, fixed with gluteraldehyde (in phosphate buffer), and gently centrifuged. Cubes (1 mm³) of the centrifuged cells were postfixed in phosphate-buffered osmium tetroxide at pH 7.3 for 1.5 hr, dehydrated in a graded series of ethanol, and embedded in an Epon-Araldite mixture. Thin sections were cut and stained with uranyl acetate and lead citrate. The sections were examined and photographed by using a RCA-EMU 3G electron microscope.

Results and Discussion

Spectral Analysis of HBB

The structural assignment of HBB was unambiguously confirmed by spectral and elemental analysis. Only two singlets of equal intensity at $\delta 7.47$ and $\delta 7.92$ were observed in the proton NMR spectrum. This pattern excludes all isomeric structures except one, HBB. This substitution pattern was further confirmed by carbon-13 NMR spectroscopy (Fig. 1): which showed bands at 122.4 ppm from TMS (C-2), 123.8 ppm (C-5), 125.9 ppm (C-4), 134.8 ppm (C-6), 136.9 ppm (C-3), and 140.2 ppm (C-1). Elemental analysis of this sample gave the following results: C, 22.96%; H, 0.64%; Br, 76.40%. These values fit extremely well with the calculated values for a hexabromobiphenyl molecule (C, 22.69%; H, 0.69%; Br, 76.08%). Mass spectral analysis (Fig. 2) showed a molecular ion at m/e 622 with a well-defined bromine isotope pattern.

Cellular Growth Patterns

The effects on cell growth of HBB and FF-1 at three concentrations and of HCB at two concentrations is depicted in Figure 3. At 75 $\mu\text{g/ml}$, HCB had a greater toxicity than either HBB or FF-1; FF-1 was more toxic than HBB, except at the 13-day time, when they were similar. At 37 $\mu\text{g/ml}$ HCB was more toxic than both HBB and FF-1, except at 13 days after treatment when they essentially were the same. The growth of HCB-treated cells was linear at 37 $\mu\text{g/ml}$ compared to the controls, whereas the growth of HBB- and FF-1 treated cells sharply de-

clined after 8 days. At 5 $\mu\text{g/ml}$, HBB stimulated cell growth after 4 and 8 days treatment; FF-1 stimulated the growth after 8 days.

Cellular Morphology

HBB-treated cells did not show the degree of postconfluence inhibition of cell division as did the controls. Cell division continued in the HBB treated cells (Fig. 4) after a monolayer had formed resulting in focal areas of greater cell density. HBB treated cells demonstrated a crowded growth pattern (Fig. 5) in contrast to control cells (Fig. 6) which maintained cell separation by inhibition of division. The FF-1 treated cells were unable to grow in a monolayer and attached poorly to the plastic (Fig. 4 and 7). Ultrastructural features of the FF-1-treated cells showed a decrease in the surface villi as compared to control cells. The decrease in microvilli and total surface villi as compared to control cells could account for the poor attachment observed. Both the HBB and the FF-1 treated cells had an increased number of lysosomes and autophagic vacuoles relative to the control cells (Figs. 8–10).

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